

Original Article

# Cysteinyl leukotriene receptor 1 mediates LTD<sub>4</sub>-induced activation of mouse microglial cells *in vitro*

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**Aim:** To investigate the roles of cysteinyl leukotriene receptors CysLT<sub>1</sub>R and CysLT<sub>2</sub>R in leukotriene D<sub>4</sub> (LTD<sub>4</sub>)-induced activation of microglial cells *in vitro*.

**Methods:** Mouse microglial cell line BV2 was transfected with pcDNA3.1(+)-hCysLT<sub>1</sub>R or pcDNA3.1(+)-hCysLT<sub>2</sub>R. The expression of relevant mRNAs and proteins in the cells was detected using RT-PCR and Western blotting, respectively. Phagocytosis was determined with flow cytometry analysis. The release of interleukin-1β (IL-1β) from the cells was measured using an ELISA assay.

**Results:** The expression of CysLT<sub>1</sub>R or CysLT<sub>2</sub>R was considerably increased in the transfected BV2 cells, and the receptors were mainly distributed in the plasma membrane and cytosol. Treatment of the cells expressing CysLT<sub>1</sub>R or CysLT<sub>2</sub>R with CysLT receptor agonist LTD<sub>4</sub> (0.1–100 nmol/L) concentration-dependently enhanced the phagocytosis, and increased mRNA expression and release of IL-1β. Moreover, the responses of hCysLT<sub>1</sub>R-BV2 cells to LTD<sub>4</sub> were significantly larger than those of hCysLT<sub>2</sub>R-BV2 or WT-BV2 cells. Pretreatment of hCysLT<sub>1</sub>R-BV2 cells with the selective CysLT<sub>1</sub>R antagonist montelukast (1 μmol/L) significantly blocked LTD<sub>4</sub>-induced phagocytosis as well as the mRNA expression and release of IL-1β, whereas the selective CysLT<sub>2</sub>R antagonist HAMI 3379 (1 μmol/L) had no such effects.

**Conclusion:** CysLT<sub>1</sub>R mediates LTD<sub>4</sub>-induced activation of BV2 cells, suggesting that CysLT<sub>1</sub>R antagonists may exert anti-inflammatory activity in brain diseases.

**Keywords:** leukotriene D<sub>4</sub>; cysteinyl leukotriene receptor; microglia; phagocytosis; IL-1β; montelukast; HAMI 3379; inflammation; brain ischemia

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## Introduction

The cysteinyl leukotrienes (CysLTs), namely leukotriene C<sub>4</sub> (LTC<sub>4</sub>), LTD<sub>4</sub> and LTE<sub>4</sub>, are potent pro-inflammatory mediators derived from the arachidonic acid 5-lipoxygenase pathway<sup>[1,2]</sup>. CysLTs are involved in various diseases including inflammation following cerebral ischemia and brain trauma<sup>[1,2]</sup>. CysLTs act on at least two G protein-coupled receptors, CysLT<sub>1</sub>R and CysLT<sub>2</sub>R<sup>[3,4]</sup>, and these receptors mediate various responses in the peripheral and central nervous systems<sup>[5,6]</sup>.

The production of CysLTs significantly increases in the brain after focal cerebral ischemia in rats, and 5-lipoxygenase inhibitors reduce the production of CysLTs and attenuate

ischemic injuries<sup>[7,8]</sup>. In addition, the expression of CysLT<sub>1</sub>R and CysLT<sub>2</sub>R increases after focal cerebral ischemia in rats<sup>[9,11]</sup>; these proteins are localized in injured neurons during the acute phase (24 h) and in proliferating microglia and astrocytes during the late phase of cerebral ischemia (3–28 d)<sup>[9,10]</sup>. Pharmacological studies show that CysLT<sub>1</sub>R antagonists (pranlukast and montelukast) and a CysLT<sub>2</sub>R antagonist (HAMI 3379) showed dose- and time-dependent protective effects against focal cerebral ischemia in rats<sup>[12–14]</sup> and in mice<sup>[15]</sup>. These findings indicate that CysLT<sub>1</sub>Rs and CysLT<sub>2</sub>Rs may play regulatory roles in acute neuronal injury as well as in astrocytosis and microgliosis in the late phase after focal cerebral ischemia.

Limited evidence from cellular studies is available regarding the involvement of CysLT<sub>1</sub>R and CysLT<sub>2</sub>R in inflammatory responses and ischemic neuronal injury. In a previous study, LTD<sub>4</sub> (an agonist of both CysLT<sub>1</sub>R and CysLT<sub>2</sub>R)

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induced CysLT<sub>1</sub>R-mediated astrocyte proliferation at lower concentrations (1–10 nmol/L) but induced CysLT<sub>2</sub>R-mediated astrocyte injury at high concentrations (100–1000 nmol/L)<sup>[16]</sup>. However, the question of whether CysLT<sub>1</sub>R and/or CysLT<sub>2</sub>R are involved in microglial activation remains to be clarified, although their mRNA expression and mediation of purine and CysLT co-release has been reported in microglia<sup>[17]</sup>.

To address these issues, we investigated the effects of overexpression of CysLT<sub>1</sub>R and CysLT<sub>2</sub>R and antagonists of these receptors on LTD<sub>4</sub>-induced activation of BV2 cells, a mouse microglial cell line<sup>[18–20]</sup>. Phagocytotic activity and the expression and release of the pro-inflammatory cytokine interleukin-1 $\beta$  (IL-1 $\beta$ ) were determined as the indicators of microglial activation in BV2 cells.

## Materials and methods

### Reagents

LTD<sub>4</sub> (Sigma-Aldrich, St Louis, MO, USA), montelukast (Merck Pharmaceutical Co, Whitehouse Station, NJ, USA) and HAMI 3379 (Cayman Chemical, Ann Arbor, MI, USA) were dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich) and diluted in culture medium before use (the concentration of DMSO was <0.001% after dilution).

### Cell culture and receptor gene transfection

BV2 cells (Chinese Academy of Sciences, Shanghai, China) were cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM; HyClone, Logan, UT, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Sijiqing Biol Inc, Hangzhou, China). The medium was renewed every two days until cell confluence. Human CysLT<sub>1</sub>R (hCysLT<sub>1</sub>R) and hCysLT<sub>2</sub>R cDNAs subcloned into pcDNA3.0 were purchased from the cDNA Resource Center, University of Missouri-Rolla (Rolla, MO, USA). The pcDNA3.0 null vector was purchased from Invitrogen (Carlsbad, CA, USA). The vectors expressing receptor cDNAs and the null vector were transfected into BV2 cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The permanently transfected BV2 cells were selected with 350  $\mu$ g/mL G418 in DMEM supplemented with 10% FBS. Single-cell subclones were isolated and plated at low density in 24-well plates such that only a few clones grew per plate and one clone grew per well. The cells were grown for over 2 months in the selection media. The transfected BV2 cells were defined as pcDNA3.0-BV2, hCysLT<sub>1</sub>R-BV2 and hCysLT<sub>2</sub>R-BV2 cells.

### Pharmacological treatment

At 24 h after seeding, the cells were exposed to LTD<sub>4</sub> (0.01–100 nmol/L), an agonist of CysLT<sub>1</sub>R and CysLT<sub>2</sub>R. The CysLT<sub>1</sub>R antagonist montelukast (1  $\mu$ mol/L) and the CysLT<sub>2</sub>R antagonist HAMI 3379 (1  $\mu$ mol/L) were added to the medium 30 min before exposure to LTD<sub>4</sub> until the end of the experiments. The concentration of both antagonists (1  $\mu$ mol/L) was confirmed to be effective in preliminary experiments.

### RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR)

At the end of treatment, total RNA was isolated from BV2 cells with TRIzol reagent (Invitrogen) according to the manufacturer's protocol<sup>[11]</sup>. For cDNA synthesis, 2  $\mu$ g of total RNA was mixed with 1 mmol/L dNTP, 0.2  $\mu$ g of a random primer, 20 U RNasin, and 200 U M-MuLV reverse transcriptase in 20  $\mu$ L of reverse transcription reaction buffer. The mixture was incubated at 42 °C for 60 min and subsequently heated at 72 °C for 10 min to deactivate the reverse transcriptase.

The primer sequences were designed using Primer Premier software, and the specificity of the oligonucleotide primers was verified using the program BLASTN. The primer sequences are as follows: hCysLT<sub>1</sub>R, forward 5'-(+)ATA GAC CAC ACG GAG AGG CAG T-3' and reverse 5'-(+)CTG CCA CAT GCC ATG ACA CTA-3'; hCysLT<sub>2</sub>R, forward 5'-(+)GCC CAC CAC CAA GGC AAT ATA-3' and reverse 5'-(+)CGT TTC CTG GCA ATG GTT CA-3'; human  $\beta$ -actin, forward 5'-(+)CTA GAA GCA TTG CGG TGG-3' and reverse 5'-(+)TGA CGG GGT CAC CCA CAC TGT GCC CAT CTA-3'; mouse IL-1 $\beta$ , forward 5'-(+)GCC CAT CCT CTG TGA CTC AT-3' and reverse 5'-(+)AGG CCA CAG GTA TTT TGT CG-3'; mouse  $\beta$ -actin, forward 5'-(+)GTC GTA CCA CAG GCA TTG TGA TGG-3' and reverse 5'-(+)GCA ATG CCT GGG TAC ATG GTG-3'.

PCR was performed using an Eppendorf Master Cycler (Eppendorf Scientific Inc, Westbury, NY, USA). The reaction conditions were set as follows: 1  $\mu$ L of the cDNA mixture was added to 20  $\mu$ L of reaction buffer containing 1.5 mmol/L MgCl<sub>2</sub>, 0.2 mmol/L deoxynucleotide triphosphates, 20 pmol/L primers and 1 U Taq DNA polymerase. The mixtures were initially heated at 94 °C for 2 min, followed by 35 cycles of 94 °C for 60 s, 52 °C for 30 s and 72 °C for 60 s followed by a final extension step of 72 °C for 10 min. With the exception of IL-1 $\beta$ , the reaction mixtures were initially heated at 94 °C for 2 min followed by 30 cycles of 94 °C for 30 s, 54 °C for 30 s and 72 °C for 60 s with a final extension step of 72 °C for 10 min. The PCR products (10  $\mu$ L) were separated by 2% agarose gel electrophoresis and visualized by ethidium bromide staining. The density of each band was measured with a UVP gel analysis system (Bio-Rad Laboratories, Hercules, CA, USA). The results are expressed as the ratio to  $\beta$ -actin.

### Western blotting analysis

The cells were washed twice with ice-cold phosphate-buffered saline (PBS) and subsequently lysed at 4 °C in lysis buffer (Kangchen Biotechnology, Shanghai, China). The lysate was obtained by centrifugation at 12000 $\times$ g at 4 °C for 30 min. Protein concentrations were determined by the Bradford assay. Protein samples (120  $\mu$ g) were subjected to Western blotting using the following antibodies: rabbit polyclonal antibodies against CysLT<sub>1</sub>R (1:500)<sup>[21]</sup> and CysLT<sub>2</sub>R (1:500)<sup>[22]</sup> and a mouse monoclonal antibody against glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 1:5000; Kangchen Biotechnology, Shanghai, China). The membranes were incubated with

the antibodies at 4°C overnight. After repeated washes, the membranes were incubated with anti-rabbit IRDye™ 700-conjugated antibody or anti-mouse IRDye™ 800-conjugated antibody (1:5000; Rockland Immunochemicals Inc, Gilbertsville, PA, USA). The immunoblots were analyzed using the Odyssey Fluorescent Scanner (LI-COR Biosciences, Lincoln, NE, USA). The protein bands were quantified using Bio-Rad Quantity One software (Bio-Rad, USA). The results are expressed as the ratios to GAPDH.

#### Immunocytochemistry

The cells cultured on coverslips (fixed with 4% paraformaldehyde) were sequentially incubated with rabbit polyclonal antibodies against CysLT<sub>1</sub>R or CysLT<sub>2</sub>R at 4°C overnight and subsequently with FITC-labeled goat anti-rabbit IgG (1:200; Chemicon, USA) at room temperature for 2 h. The nuclei were stained in PBS containing 1 µg/mL 4',6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich) for 1 min. Finally, the cells were examined under a fluorescence microscope (Olympus BX51, Japan).

#### Phagocytosis assay

The phagocytosis assay was performed as previously described<sup>[23]</sup>. In brief, cells were seeded on 35-mm Petri dishes at a density of  $1.5 \times 10^5$  cells/dish. LTD<sub>4</sub> (0.01–100 nmol/L) was added to the culture for 3 h in the presence or absence of CysLT receptor antagonists. One hour before cell harvest, fluorescent microspheres (red, diameter 1 µm, Invitrogen) were added at a density of  $6 \times 10^7$  particles/dish. The cells were then washed thoroughly with PBS containing 1% bovine serum albumin and detached by trypsinization. Then, the cells were quenched with 1% FBS and subjected to FACScan analysis using a FC500MCL flow cytometer (Beckman Coulter Inc, USA). Fluorescence intensity was detected in the FL-2 channel (564–606 nm) and reflected the phagocytic activity of the cells. The results are expressed as phagocytic index (percentage of the control).

#### Measurement of interleukin-1β (IL-1β)

According to a previously reported method<sup>[18]</sup>, cells were seeded into 24-well culture plates at  $2 \times 10^5$  cells/well in 0.5 mL standard culture medium for 24 h. After treatment with LTD<sub>4</sub> and the antagonists, cell-free supernatants were stored at -80°C. Released IL-1β was assayed in the supernatants using a commercial IL-1β enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems Inc, USA) according to the manufacturer's instructions and calculated as pg/mL.

#### Statistical analysis

The data were analyzed with the GraphPad Prism Software (version 5.01; GraphPad Software Inc, San Diego, CA, USA) and are presented as the mean±SEM. To compare differences, one-way analysis of variance (ANOVA) and Dunnett's test or Dunn's test were performed. A value of  $P < 0.05$  was considered statistically significant.

## Results

### Gene expression of hCysLT<sub>1</sub>R and hCysLT<sub>2</sub>R

First, we confirmed successful transfection of cells with hCysLT<sub>1</sub>R and hCysLT<sub>2</sub>R. The expression and subcellular distribution of these receptors were determined by RT-PCR, Western blotting and immunocytochemistry. After permanent transfection with hCysLT<sub>1</sub>R (Figure 1A) or hCysLT<sub>2</sub>R (Figure 1B), the mRNA and protein expression of these receptors increased in BV2 cells, and both receptors were mainly distributed in the plasma membrane and cytosol.

### The LTD<sub>4</sub>-enhanced phagocytosis of BV2 cells is mediated by CysLT<sub>1</sub>R

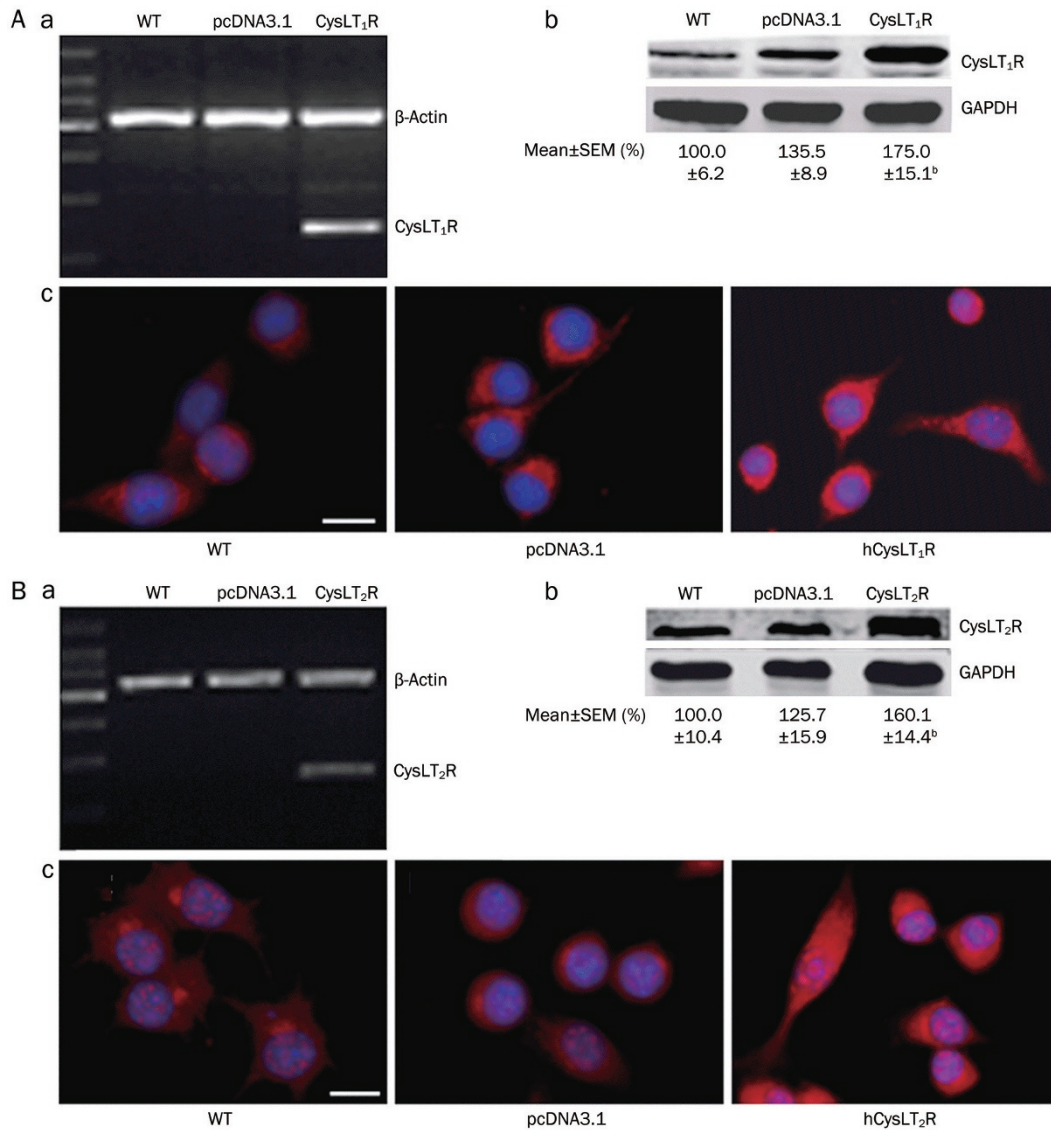
To examine phagocytotic activity, latex microparticles were employed as a tracer. LTD<sub>4</sub> (0.1–100 nmol/L) significantly increased the phagocytotic activity of BV2 cells in a concentration-dependent manner. LTD<sub>4</sub> (100 nmol/L for 3 h) increased phagocytic activity to a significantly greater extent in hCysLT<sub>1</sub>R-BV2 cells (218.8%) than in WT-BV2 and hCysLT<sub>2</sub>R-BV2 cells (158.4% and 174.0%), indicating that LTD<sub>4</sub> was able to induce the activation of phagocytosis in BV2 cells, and hCysLT<sub>1</sub>R-BV2 cells were more sensitive to LTD<sub>4</sub> (Figure 2A and 2B).

To explore the receptor subtype responsible for LTD<sub>4</sub>-enhanced phagocytosis, we assessed the effects of the CysLT<sub>1</sub>R antagonist montelukast and the CysLT<sub>2</sub>R antagonist HAMI 3379. Montelukast (1 µmol/L) and HAMI 3379 (1 µmol/L) themselves did not affect the phagocytosis of BV2 cells (Figure 3). Montelukast, but not HAMI 3379, significantly attenuated LTD<sub>4</sub>-induced phagocytosis in hCysLT<sub>1</sub>R-BV2 cells (Figure 3). These findings indicate that LTD<sub>4</sub>-enhanced phagocytosis might be regulated by CysLT<sub>1</sub>R but not by CysLT<sub>2</sub>R in BV2 cells.

### LTD<sub>4</sub>-induced upregulation of IL-1β mRNA and IL-1β release are mediated by CysLT<sub>1</sub>R

Because the release of pro-inflammatory cytokines is an important functional change during microglial activation, we determined whether LTD<sub>4</sub> increases IL-1β production in BV2 cells and identified the CysLTR subtype involved. The results showed that IL-1β mRNA expression was significantly increased by 100 nmol/L LTD<sub>4</sub> in all types of BV2 cells, and this increase was significantly higher in hCysLT<sub>1</sub>R-BV2 cells (Figure 4A and 4B). Pretreatment with the CysLT<sub>1</sub>R antagonist montelukast (1 µmol/L) decreased LTD<sub>4</sub>-upregulated expression of IL-1β mRNA to the control level (Figure 4A and 4B). However, the CysLT<sub>2</sub>R antagonist HAMI 3379 (1 µmol/L) did not reduce the LTD<sub>4</sub>-induced increase in IL-1β mRNA expression (Figure 4B). These results suggest that CysLT<sub>1</sub>R might be involved in LTD<sub>4</sub>-induced upregulation of IL-1β mRNA.

Finally, we determined whether LTD<sub>4</sub> regulates IL-1β release in BV2 cells and whether CysLT receptor antagonists affect the release of IL-1β. The ELISA results reveal that IL-1β release was increased two-fold after 3 h of exposure to 100 nmol/L LTD<sub>4</sub>, but not after 1 h of exposure, in hCysLT<sub>1</sub>R-BV2



**Figure 1.** Identification of hCysLT<sub>1</sub>R and hCysLT<sub>2</sub>R expression after stable transfection into BV2 cells. The expression of hCysLT<sub>1</sub>R (A) and hCysLT<sub>2</sub>R (B) was assessed by RT-PCR (a), Western blotting (b) and immunostaining (c). Protein expression data (b) are reported as the mean±SEM (n=4. <sup>b</sup>P<0.05, <sup>c</sup>P<0.01 vs WT-BV2 cells) and were analyzed by one-way ANOVA. Scale bar=50 μm.

cells (Figure 5A and 5B). Montelukast significantly decreased LTD<sub>4</sub>-induced IL-1β release in hCysLT<sub>1</sub>R-BV2 and hCysLT<sub>2</sub>R-BV2 cells; however, HAMI 3379 did not affect the release of IL-1β (Figure 6).

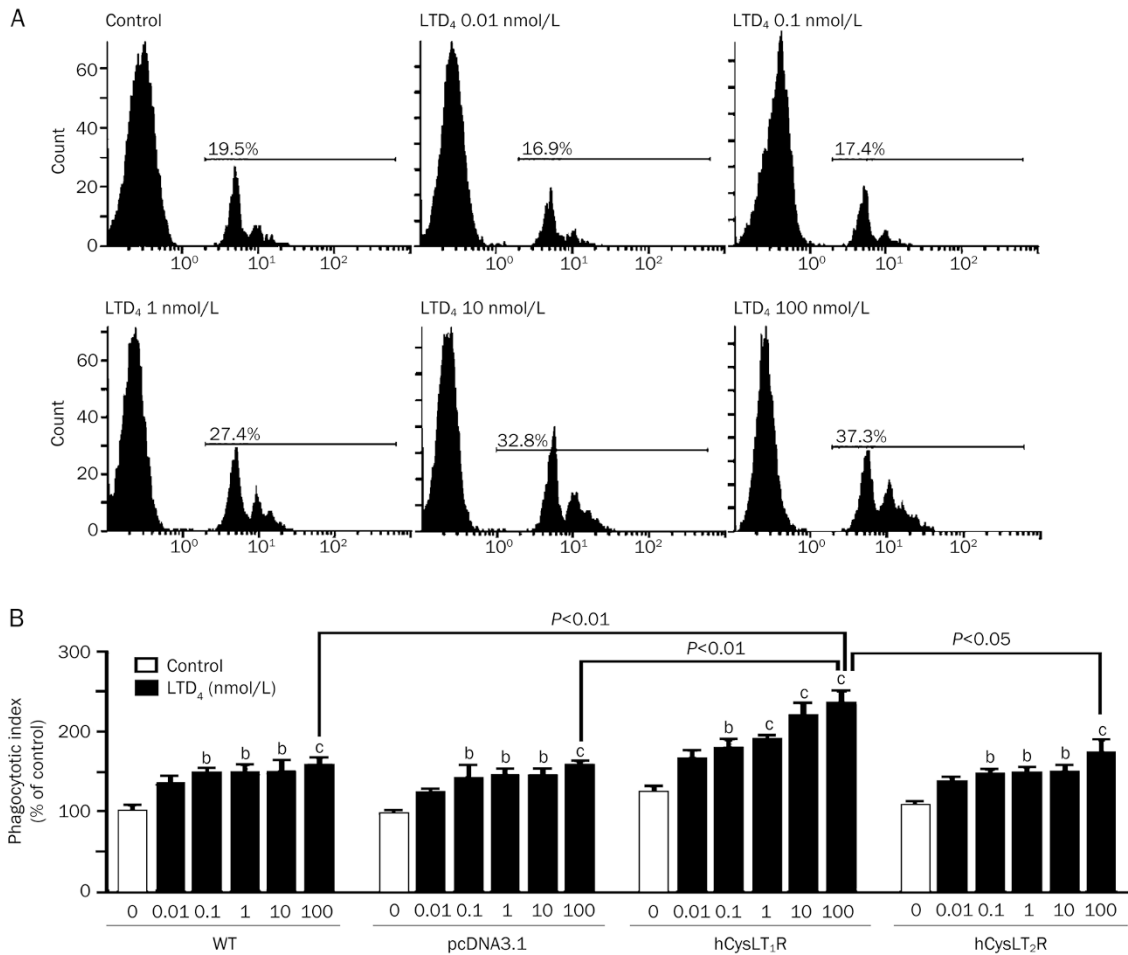
## Discussion

In the present study, we demonstrated that CysLT<sub>1</sub>R mediated the activation of BV2 microglial cells. Our results revealed that overexpression of CysLT<sub>1</sub>R increased LTD<sub>4</sub>-enhanced phagocytic activity as well as the expression and release of the inflammatory cytokine IL-1β. The pharmacological effects of CysLT<sub>1</sub>R and CysLT<sub>2</sub>R antagonists confirmed the critical role of CysLT<sub>1</sub>R. Consistent with our results, LTD<sub>4</sub> has been

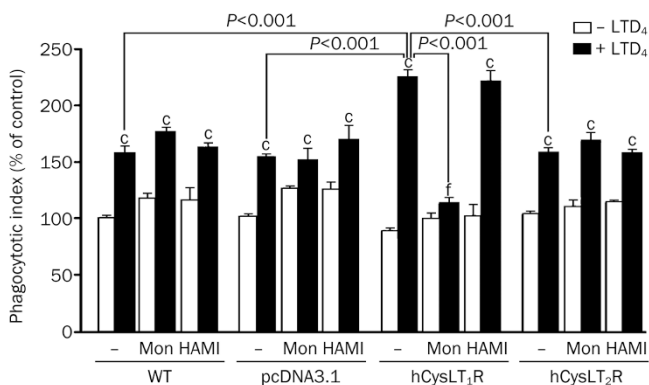
shown to enhance Fcγ receptor-induced phagocytosis of alveolar macrophages, and this enhancement was abolished by the CysLT<sub>1</sub>R antagonist MK 571<sup>[24]</sup>. LTD<sub>4</sub> also induced CysLT<sub>1</sub>R-mediated IL-1β expression and release in rat vascular smooth muscle cells<sup>[25]</sup> and cerulein-injured rat pancreas<sup>[26]</sup>.

We found that wild-type BV2 cells express CysLT<sub>1</sub>R and CysLT<sub>2</sub>R, which is supported by previous findings that primary microglia express CysLT<sub>1</sub>R and CysLT<sub>2</sub>R mRNAs<sup>[17]</sup>. Overexpression of CysLT<sub>1</sub>R or CysLT<sub>2</sub>R altered the pharmacological responses of BV2 cells to the agonist and antagonists. LTD<sub>4</sub> is a full agonist for CysLT<sub>1</sub>R and CysLT<sub>2</sub>R, with EC<sub>50</sub> values of 4.9 nmol/L and 14.4 nmol/L, respectively, in calcium flux assays<sup>[27, 28]</sup>. Thus, LTD<sub>4</sub> at 100 nmol/L, which was the





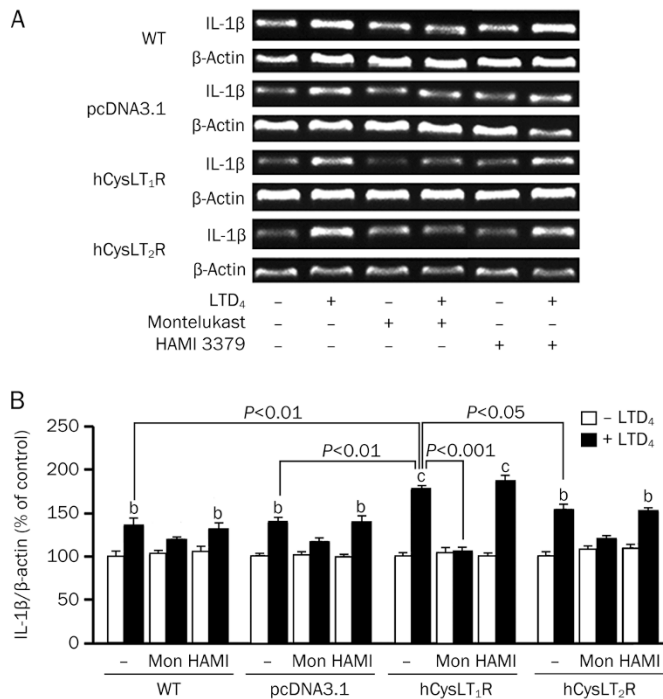
**Figure 2.** Effect of LTD<sub>4</sub> on BV2 microglial phagocytosis. (A) Flow cytometry revealed that exposure to various concentrations of LTD<sub>4</sub> for 3 h enhanced phagocytosis. (B) LTD<sub>4</sub> increased phagocytotic activity in a concentration-dependent manner, and hCysLT<sub>1</sub>R-BV2 cells were more sensitive than other types of BV2 cells. The data are reported as the mean±SEM [*n*=6. <sup>b</sup>*P*<0.05, <sup>c</sup>*P*<0.01 vs control (0 nmol/L LTD<sub>4</sub>)] and were analyzed by one-way ANOVA.



**Figure 3.** Effects of montelukast and HAMI 3379 on LTD<sub>4</sub>-induced phagocytosis. Montelukast blocked the amplified response to LTD<sub>4</sub> in hCysLT<sub>1</sub>R-BV2 cells but not in other types of BV2 cells, whereas HAMI 3379 did not show any effect. The data are reported as the mean±SEM [*n*=6. <sup>a</sup>*P*<0.01 vs control (0 nmol/L LTD<sub>4</sub>). <sup>b</sup>*P*<0.05, <sup>c</sup>*P*<0.01 vs LTD<sub>4</sub> alone in each cell type] and were analyzed by one-way ANOVA.

main stimulating condition in our experiments, can stimulate both subtypes. All the LTD<sub>4</sub>-evoked responses (phagocytosis, IL-1β expression and release) were more potent in BV2 cells overexpressing hCysLT<sub>1</sub>R than in other cell types. The effects of antagonists further confirmed the role of CysLT<sub>1</sub>R. Montelukast obviously attenuated all the amplified LTD<sub>4</sub>-evoked responses in hCysLT<sub>1</sub>R-BV2 cells, although it had no significant effect in WT-BV2 cells. However, the selective CysLT<sub>2</sub>R antagonist HAMI 3379 (1 μmol/L) had no effect on LTD<sub>4</sub>-induced responses. Thus, CysLT<sub>1</sub>R may be the major regulator in BV2 microglial activation.

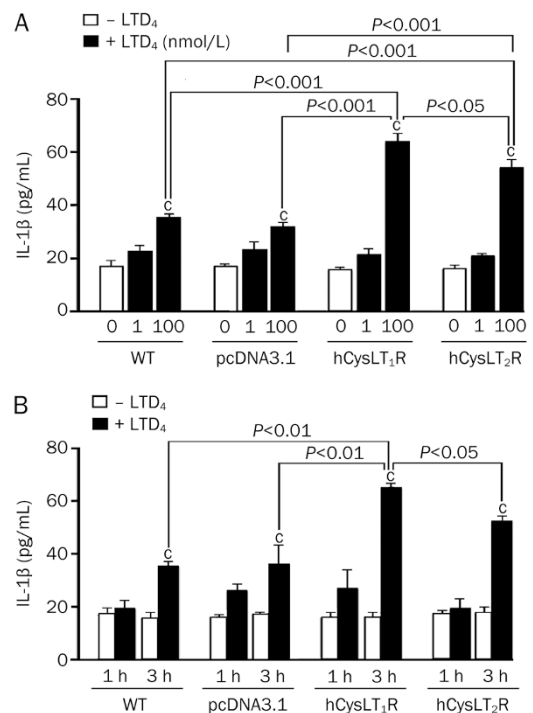
On the other hand, the role of CysLT<sub>2</sub>R remains to be addressed. Our results showed that LTD<sub>4</sub> did not affect phagocytosis but induced greater IL-1β release in hCysLT<sub>2</sub>R-BV2 cells than in WT-BV2 cells. HAMI 3379 did not inhibit LTD<sub>4</sub>-induced IL-1β release or other responses, whereas montelukast partially attenuated LTD<sub>4</sub>-induced IL-1β expression and release, but not phagocytosis, in hCysLT<sub>2</sub>R-BV2 cells. The possible explanation for this result is that overexpressed



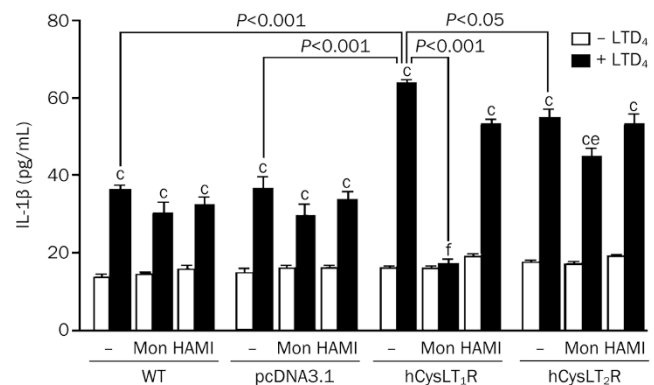
**Figure 4.** Effects of montelukast and HAMI 3379 on LTD<sub>4</sub>-induced upregulation of IL-1β mRNA expression in BV2 cells. (A) mRNA expression of IL-1β after exposure to 100 nmol/L LTD<sub>4</sub> with or without antagonists for 3 h. (B) The LTD<sub>4</sub>-induced upregulation of IL-1β mRNA expression was greater in hCysLT<sub>1</sub>R-BV2 cells than in other types of cells. Montelukast blocked the upregulation in each BV2 cell type, especially in hCysLT<sub>1</sub>R-BV2 cells. The data are reported as the mean±SEM ( $n=6$ ). <sup>b</sup> $P<0.05$ , <sup>c</sup> $P<0.01$  vs 0 nmol/L LTD<sub>4</sub>) and were analyzed by one-way ANOVA.

CysLT<sub>2</sub>R may potentiate the CysLT<sub>1</sub>R response through unknown interactions. It has been reported that heterodimers of CysLT<sub>1</sub>R and CysLT<sub>2</sub>R exist in intestinal epithelial cells and mast cells, and these heterodimers modulate the cell proliferative responses of CysLT<sub>1</sub>R<sup>[29, 30]</sup>. However, the question of whether these heterodimers exist and interact in BV2 cells requires further investigation. Moreover, the responses mediated by CysLT<sub>2</sub>R and CysLT<sub>1</sub>R may vary in different experiments. We recently reported that CysLT<sub>2</sub>R plays a major regulatory role in the activation of rat primary microglia (phagocytosis and cytokine release), whereas CysLT<sub>1</sub>R only regulates cytokine release from microglia<sup>[31]</sup>. These findings reflect differences between species (rat and mouse) and cell types (primary microglia and BV2 cells<sup>[18]</sup>); therefore, the roles of CysLT<sub>2</sub>R in BV2 cell activation should be further clarified.

IL-1β is one of the pro-inflammatory cytokines produced in microglial cells<sup>[32, 33]</sup> and is involved in various peripheral and central nervous system diseases<sup>[34-37]</sup>. Thus, this cytokine is usually used as an inflammatory marker. We chose IL-1β to serve as an index of cytokine release because its change in expression and release after exposure to LTD<sub>4</sub> was more stable than that of other cytokines in preliminary experiments.



**Figure 5.** LTD<sub>4</sub>-induced IL-1β release from BV2 cells. IL-1β in the medium of BV2 cell cultures was measured by ELISA. LTD<sub>4</sub> (100 nmol/L) did not affect the release after 1 h of exposure; however, the release was increased after 3 h of exposure. The release of IL-1β after exposure to 100 nmol/L LTD<sub>4</sub> for 3 h was significantly higher in hCysLT<sub>1</sub>R-BV2 cells. The data are reported as the mean±SEM ( $n=6$ ). <sup>c</sup> $P<0.01$  vs 0 nmol/L LTD<sub>4</sub>) and were analyzed by one-way ANOVA.



**Figure 6.** Effects of montelukast and HAMI 3379 on LTD<sub>4</sub>-induced IL-1β release from BV2 cells. LTD<sub>4</sub> (100 nmol/L) increased IL-1β release from all types of BV2 cells. hCysLT<sub>1</sub>R-BV2 cells released more IL-1β than other types of BV2 cells after exposure to LTD<sub>4</sub>, and hCysLT<sub>2</sub>R-BV2 cells released more than WT-BV2 and pcDNA3.1-BV2 cells ( $P<0.05$ ). Montelukast blocked the potent response to LTD<sub>4</sub> in hCysLT<sub>1</sub>R- and hCysLT<sub>2</sub>R-BV2 cells, whereas HAMI 3379 did not show any effects. The data are reported as the mean±SEM ( $n=6$ ). <sup>e</sup> $P<0.01$  vs 0 nmol/L LTD<sub>4</sub>, <sup>f</sup> $P<0.05$ , <sup>g</sup> $P<0.01$  vs LTD<sub>4</sub> alone in each cell type) and were analyzed by one-way ANOVA.

We found that LTD<sub>4</sub> enhanced IL-1 $\beta$  mRNA expression and increased release by activating CysLT<sub>1</sub>R. Consistently, it has been reported that activated BV2 cells release IL-1 $\beta$ <sup>[18]</sup>, and CysLT<sub>1</sub>R mediates LTD<sub>4</sub>-elicited IL-4 release from cord blood-derived human eosinophils<sup>[38]</sup>. Therefore, CysLT<sub>1</sub>R is an important regulator of inflammatory cytokine release in microglial cells in addition to its role as a regulator of microglial phagocytosis. The released cytokines, in turn, regulate the CysLT receptor; for example, IL-1 $\beta$ , interferon- $\gamma$  (IFN- $\gamma$ ) and transforming growth factor  $\beta$  (TGF- $\beta$ ) enhance the expression of CysLT<sub>1</sub>R<sup>[2]</sup>. However, the interactions between CysLT<sub>1</sub>R and IL-1 $\beta$  during microglia activation remain to be explored.

Moderately activated microglia can play a neuroprotective role due to their ability to remove dead cells and to release trophic factors<sup>[39]</sup>, which facilitates the reorganization of neuronal circuits and the triggering of repair<sup>[40]</sup>. However, over-activated microglia injure neurons by releasing detrimental factors<sup>[41, 42]</sup> such as cytokines (eg, IL-1 $\beta$  and TNF- $\alpha$ ) and nitric oxide (NO)<sup>[18]</sup> and by activating inflammation-related kinases (eg, JNK and p38) and transcription factors (eg, c-JUN and NF- $\kappa$ B)<sup>[43]</sup>. As the most important inflammatory mediators, CysLTs may, through the mediation of their receptors, participate in the activation of microglia, an inflammatory event that occurs in the central nervous system after brain injury. Our previous studies demonstrated that both CysLT<sub>1</sub>R and CysLT<sub>2</sub>R are upregulated in proliferating microglia surrounding the ischemic area in the brains of rats with focal cerebral ischemia<sup>[9, 10]</sup>; however, the roles of these proteins in the regulation of microglial function have not been revealed. The present study has demonstrated the role of CysLT<sub>1</sub>R in the activation of BV2 microglial cells.

The responses of BV2 cells are similar to those of primary microglia in most aspects but different in other aspects<sup>[18]</sup>; therefore, our findings largely imply a regulatory role for CysLT<sub>1</sub>R in microglial activation after brain injury. These findings can also partially explain the neuroprotective effects of CysLT<sub>1</sub>R antagonists in subacute or chronic brain ischemia<sup>[10, 44, 45]</sup>. However, the roles of CysLT<sub>1</sub>R and CysLT<sub>2</sub>R should be investigated in primary microglia or *in vivo* animal experiments.

In summary, our findings indicate that CysLT<sub>1</sub>R mediates the activation of BV2 microglial cells, suggesting that its antagonists will be effective for inhibiting inflammation after brain injury. However, the detailed mechanisms underlying this process as well as the role of CysLT<sub>2</sub>R remain to be investigated.

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### Author contribution

Shu-ying YU, Xia-yan ZHANG, Xiao-rong WANG, Dong-

min XU, and Lu CHEN performed the experiments; Li-hui ZHANG, San-hua FANG, Yun-bi LU, and Wei-ping ZHANG supervised all aspects of the research and revised the manuscript; and Shu-ying YU and Er-qing WEI prepared the manuscript.

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